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IMMUNOHISTOCHEMICAL LOCALIZATION OF THE NEUROPEPTIDE RFAMIDE IN THE HYPOSTOMAL NERVE NET OF *HYDRA VULGARIS*

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BY

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ABSTRACT

This study examined the distribution and localization of the neuropeptide arginine-phenylalanine-amide (RFamide) in the hypostomal and tentacular nerve net of the cnidarian polyp *Hydra vulgaris*. Immunohistochemical techniques were used to visualize three RFamide-positive distinct nerve ring structures, nerves and neurites throughout the ectoderm of the hypostome and tentacles of ablated hypostomes. Two RFamide-positive circumhypostomal nerve rings are reported here: an inner ring located near the mouth and an outer ring located beneath the tentacle attachment sites. Both rings are consistent with rings labeled with anti-α-tubulin; the outer ring is also coincident with a $GABA_B$ receptor-antibody-labeled ring previously described (Hufnagel and Kass-Simon, manuscript in preparation). A third, previously undescribed, set of nerve ring structures are reported, these are circumtentacular nerve rings located around the base of each tentacle. These circumtentacular rings were situated at the tentacle-hypostome junctions. Comparison preparations using anti-α-tubulin antibodies also showed circumtentacular ring structures. A continuous RFamide-labeled nerve net was seen throughout the hypostome and tentacles. RFamide labeled nerves were observed to be consistently associated with the nematocytes; this was especially apparent in the battery cell complexes within the tentacles. These results expand on the understanding of the role of RFamide in the chemical neurotransmission of hydra and suggest that RFamide leads to tentacular contractions during hydra feeding behaviors.

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PREFACE

This thesis is being submitted in manuscript format. It is composed of one manuscript and three appendices. The title of the manuscript is "Immunohistochemical localization of the neuropeptide RFamide in the hypostomal nerve net of *Hydra vulgaris*". This manuscript will be submitted to *Tissue& Cell*, with co-authors Gabriele Kass-Simon and Linda Hufnagel.

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"IMMUNOHISTOCHEMICAL LOCALIZATION OF THE NEUROPEPTIDE RFAMIDE IN THE HYPOSTOMAL NERVE NET OF *HYDRA VULGARIS*"

BY

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is formatted and prepared for submission to *Tissue& Cell*.

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ABSTRACT

This study examined the distribution and localization of the neuropeptide arginine-phenylalanine-amide (RFamide) in the hypostomal and tentacular nerve net of the cnidarian polyp *Hydra vulgaris*. Immunohistochemical techniques were used to visualize three RFamide-positive distinct nerve ring structures, nerves and neurites throughout the ectoderm of the hypostome and tentacles of ablated hypostomes. Two RFamide-positive circumhypostomal nerve rings are reported here: an inner ring located near the mouth and an outer ring located beneath the tentacle attachment sites. Both rings are consistent with rings labeled with anti-α-tubulin; the outer ring is also coincident with a $GABA_B$ receptor-antibody-labeled ring previously described (Hufnagel and Kass-Simon, manuscript in preparation). A third, previously undescribed, set of nerve ring structures are reported, these are circumtentacular nerve rings located around the base of each tentacle. These circumtentacular rings were situated at the tentacle-hypostome junctions. Comparison preparations using anti-α-tubulin antibodies also showed circumtentacular ring structures. A continuous RFamide-labeled nerve net was seen throughout the hypostome and tentacles. RFamide labeled nerves were observed to be consistently associated with the nematocytes; this was especially apparent in the battery cell complexes within the tentacles. These results expand on the understanding of the role of RFamide in the chemical neurotransmission of hydra and suggest that RFamide leads to tentacular contractions during hydra feeding behaviors.

INTRODUCTION

Hydra species have the simplest body plans among cnidarian species. However, contrary to previous views, hydra's nervous system is not a simple nerve net as originally described by Schneider (1890) and Hadzi (1909), but appears to be organized into pharmacologically differentiable regions (Koizumi 2002), which contain all the classical neurotransmitters of more complex systems (Kass-Simon and Pierobon 2007).

Immunostaining using antibodies made against tubulin and neuropeptides has identified distinct subsets of neurons with specific spatial distributions (Grimmelikhuijzen 1985). It has also been shown that neurons expressing different neuropeptides are distributed in a polarized way with respect to the body axis of the hydra (Koizumi et al. 2004). In addition to the nerve net, nerve rings have been identified, and described as putative integrative centers where activity patterns that result in coordinated behavior are generated (Grimmelikhuijzen 1985; Koizumi et al. 1992).

The genus *Hydra* has been classified into four groups: the 'vulgaris group' (common hydra), the 'oligactis group' (stalked hydra), the 'viridissima group' (green hydra) and the 'braueri group' (gracile hydra). Color, presence or absence of a body stalk, nematocyst shape, and the order of appearance of tentacles on the new buds are characteristics used to differentiate between the groups of genera (Campbell 1987). The RFamide neuropeptide family has been extensively studied in the four groups, all

of which contain RFamide positive nerve cells in the hypostome (Koizumi 2007). Yet, there have been varying morphological descriptions of the distributions and centralizations of these RFamide positive nerve cells.

Thus far, immunohistochemical studies with anti-RFamide antibodies have shown the presence of circumhypostomal nerve rings in species categorized as stalked and gracile hydra (Koizumi 2007), whereas similar studies on *Hydra vulgaris (*common hydra) did not reveal a nerve ring; only that two neuronal centralizations occur, in the ectoderm of the hypostome and the lower peduncle (Grimmelikhuijzen 1985). Specifically, RFamide positive sensory neurons were found to be located in the ectoderm of the hypostome, around the mouth opening (Grimmelikhuijzen 1985; Plickert 1989; Koizumi et al. 1992) and RFamide positive ganglion cells were identified in the tentacles and peduncle, as well as in the hypostome (Grimmelikhuijzen 1985; Koizumi 2002).

However, the immunohistochemical evidence of the centralizations of RFamide in *Hydra vulgaris* did not correlate with electrophysiological and ultrastructural evidence. Previous electrophysiological studies on *H. vulgaris* provided evidence for an ectodermal pacemaker system, responsible for the periodic body-column contractions, which appeared to be comprised of a set of several interconnected neuronal loci, situated at or near the base of tentacle insertions (Passano and McCullough 1964; Kass-Simon 1972, 1973) where no RFamide positive neurons had been identified. The impulses of this pacemaker system are affected by the amino acid transmitters, glutamate and gamma-Aminobutyric acid (GABA) (Kass-Simon et al. 2003), acetylcholine antagonists (Kass-Simon and Passano 1978) and

glycine (Ruggieri et al. 2004). Recent immunohistochemical evidence indicates that a loose ring of neurons comprised of a set of several interconnected neuronal loci, is situated at or near the base of tentacle insertions which label with anti-$GABA_B$ receptor antibodies (Hufnagel and Kass-Simon, MS in preparation). Locations of high densities of neuronal cell bodies were found in a similar location as the predicted ectodermal pacemaker system of *H. vulgaris*, in strains of *H. vulgaris* and *H. oligactis.* Researchers using electron microscopy described high densities of neuronal cell bodies on the apical side of the base of each tentacle in *H. littoralis* (Kinnamon and Westfall 1981), which is now recognized to be a strain of *H. vulgaris* (Campbell 1983). Concentrations of neuronal cell bodies in *Pelmatohydra robusta* (now considered to be a strain of *H. oligactis*) (Matsuno and Kageyama 1984) were also observed between tentacles. In *P. robusta*, Matsuno and Kageyama also observed a nerve ring encircling the hypostome. This ring consisted of clusters of ganglion cells, thick bundles of many neurites connecting these clusters, and a small number of individual ganglion cells located along the bundles. Furthermore, they found that circumferential bundles of neurites were located in the region of high neuronal cell body density.

Recently, immunohistochemical experiments using antibodies against anti-α tubulin, a protein that is abundant in nerve cell bodies and neurites, provided evidence for two hypostomal nerve rings in *H. vulgaris*; the proximal nerve ring and the distal nerve ring (Hufnagel and Kass-Simon, MS in preparation). The location of the proximal tubulin nerve ring correlates with the ectodermal pacemaker system that had been characterized through electrophysiological experiments (Passano and

McCullough 1964, Kass-Simon 1972, 1973), thus providing a morphological basis for the physiological observations. The location of the distal tubulin nerve ring of *H. vulgaris* appears to be similar to the location of the RFamide positive ring described in gracile and stalked hydras (Koizumi 2007; Grimmelikhuijzen 1985). Coincident with the proximal tubulin nerve ring, a ring that labels with anti-$GABA_B$ receptor antibody was found (Hufnagel and Kass-Simon, MS in preparation). The question that arose, therefore, and which was addressed in this study, was whether either the proximal, GABA receptor associated ring or the distal tubulin labeled ring also labeled positively with the RFamide antibody. The goal of the present study, therefore, was to examine by immunohistochemistry whether the proximal, the distal or both hypostomal nerve rings of *H. vulgaris* might contain RFamide.

MATERIALS AND METHODS

Animal Cultures. All experiments were carried out on *Hydra vulgaris,* incubated at 19+/-2 ^{o}C in glass culture dishes containing bicarbonate versene culture medium (BVC; 0.1mM $NaHCO_3$, 1.0 mM $CaCl_2$, and 0.01 mM EDTA in distilled water, pH 7.0 +/- 0.2; Kass-Simon et al. 2003). Animals were fed with *Artemia salina* nauplii on alternate days. The culture solution was changed 45 minutes after feeding. Experimental hydras were chosen from 48 hour starved animals.

Immunohistochemistry: Individual hydra were transferred to the middle of a PAP-pen square on an agar-coated slide and relaxed using dilute (1/1000 w/v) menthol in BVC solution. Each animal was transected directly underneath the tentacle/ hypostome region using a scalpel (# 11 blade); the body was discarded, leaving only the tentacle and hypostome on the slide. The tissue was treated with dissociation medium (1 part glycerol, 1 part acetic acid, 7 parts deionized water), followed by Zamboni's fixative (Zamboni et al. 1967) and left overnight at 4 ^{o}C in moist chambers. The following day, the slide-mounted preparations were washed with phosphate buffered saline (PBS), treated with 0.4 M glycine for 2 hours, washed with modified PBS solution containing 1% bovine serum albumin (MPBS/BSA; 1% bovine serum albumin, 0.2% Tween 80, 0.05% sodium azide, 0.1% polyethylene glycol 20 and 0.02% KCl; Erskine 1989) and subsequently incubated in primary antibody (anti-RFamide mouse monoclonal antibody received from O. Koizumi, Fukuoka Women's University, Japan), in moist chambers at room temperature (RT) overnight. The next day, the hydra were washed

twice with MPBS/BSA and incubated overnight at RT in a 1/500 dilution of goat anti-mouse secondary antibody tagged with Alexa488 (Molecular Probes/Invitrogen, Eugene, OR). Hydra slides were washed twice in MPBS/BSA; then Prolong Gold mounting medium (Molecular Probes/Invitrogen, Eugene, OR) was added to each slide, and cover slips were applied. Once dry, the coverslips were sealed with nail polish and stored at -18 ^{o}C until examined. Negative controls, in which the primary antibody was omitted, were run in all experiments. Specific binding of primary antibodies was demonstrated by the fact that negative control slides, lacking primary antibody, revealed only very low-level background fluorescence, which was not detectable under the imaging conditions used for the experimental slides. The RFamide mouse monoclonal antibodies were produced by Dr. Osamu Koizumi and specificity was confirmed using ELISA. Experiments were run using anti-α-tubulin (Sigma Clone B512) primary antibodies to compare with the topology of the RFamide labeled nerve structures.

Imaging and Analysis. Slides were evaluated and digital photographs taken using a Carl Zeiss LSM700 confocal system equipped with multiple lasers and excitation and emission fluorescence filters and ZENBlack imaging software. Further analysis of slides were done using ImageJ/ FIJI software. The AlexaFluor488 labeled samples were imaged using FITC filters. Photographs were taken using Plan Neofluar 20X and 40X objectives. Z-stack series and tile scans were acquired. Z-stack series were done by obtaining multiple images sequentially at different planes of focus. Tile scans were done by acquiring multiple partial images which were stitched together with

ZENBlack software to form one complete image of the whole object (Berthiaume and Morgan 2010); thus making it possible to see larger fields of view with a high magnification objective. Pairs of differential interference contrast (DIC) and fluorescence images were taken of each region photographed, at the same focal plane. DIC imaging takes advantage of differences in the light refraction of samples, which allows even non-fluorescent labeled samples to become visible during evaluation (Mehrotra 2009). Brightness and contrast were adjusted to improve visualization.

RESULTS

The nerve net in whole mounts of whole and partially dissociated tentacles and hypostomes of *Hydra vulgaris* were labeled with monoclonal anti-RFamide antibodies. Staining was seen in a variety of nerve cells throughout the ectoderm of the hypostome and tentacle. Staining was not observed in the endoderm. An overview of hydra's morphology is depicted in figure 1. Experiments were run using anti-α-tubulin primary antibodies to compare with the RFamide labeled nerve structures (Fig 2). The RFamide-positive labeling was cell-type specific: neuronal cells were readily seen labeled but epithelial cells were not (Fig 3). RFamide positive labeling distribution and appearance are described below (Fig 4).

Nerve rings

A distinct RFamide-positive nerve ring was observed in the ectoderm in the apical region of the hypostome. Circumferentially running nerve fibers appeared to make up the distinct nerve ring (Fig 5A). Many sensory cell bodies located near the ring had neurites feeding into the ring (Fig 5B). In z-stacks it was possible to follow a neurite emanating from a sensory cell body into the nerve ring. Sensory cell labeling appeared to be localized within the cell body, not evenly distributed throughout the entire cell (arrows in Fig 5B). Stenotele nematocytes were seen intermittently to be in close proximity to the nerve ring (Fig 5B). This ring corresponded to the location and appearance of the anti-α-tubulin stained distal ring described by Hufnagel and Kass-Simon (manuscript in preparation) and seen in our current experiments (Fig 2).

A second RFamide-positive nerve ring was observed in the ectoderm in the basal region of the hypostome; located beneath the insertion of the tentacles (Fig 6). This ring appeared to consist of bipolar nerve cells with long neurites showing punctate RFamide labeling. Neurites, resembling pearls strung on a necklace, were also found to be associated with nematocytes (Fig 7B).

RFamide-positive circumtentacular nerve rings were observed at the junction of the tentacles and hypostome. The rings consisted of tightly packed nerve cell bodies and neurites that encircled the base of each tentacle (Fig 8). Similar rings were seen in preparations labeled with anti-α-tubulin (Fig 2). Neurites were observed to run between the hypostome and the tentacle and connect with the circumtentacular nerve rings. It was not possible to identify the type or types of nerve cells that make up these rings.

Structures

Hypostome

A dense nerve plexus labeled in the mouth area (Fig 9), which became more diffuse as the distance from the apex increased. This dense area was rich in sensory cell bodies and their neurites. The sensory cell processes were seen extending to the surface of the ectoderm (arrows in Fig 9). Labeling in the neurites which radiated down the hypostome towards the base of the tentacles was punctate in appearance. RFamide labeling within the cells did not appear to be distributed evenly; instead it appeared to be localized in the anterior and circumferential portions of the cell (Fig 9). Nerve cells which appeared to be ganglion cells with multiple neurites, were seen to

be intermingled with some of the sensory cells throughout the hypostome, except in the immediate vicinity of the mouth, where only sensory cells were observed (Fig 10).

In the basal region of the hypostome, many brightly labeled developing nematoblasts were seen (Fig 11). They existed in nests, usually containing at least 8 or more cells. Tripolar ganglion cells were observed in the basal part of the hypostome with punctate-labeled neurites emanating from them (Fig 12). The neurites punctate labeling resembled dotted lines.

Tentacles

The RFamide positive nerve plexus was seen extending from the hypostome into the tentacles. Labeling at the base of the tentacles was prominent. While labeling of neurites could be seen throughout the length of the tentacles, neurites at the base of the tentacles appeared to be more densely labeled (Fig 13A). Immunoreactive tripolar ganglion cells were distributed throughout the tentacles (Fig 13B). Especially striking, was the great number of stenoteles closely associated with nerve cells (Fig 14A). Nerve cells were seen associated with the nematocytes located within battery cells (Fig 15B). The desmonemes in the battery cell complex also appeared to be closely associated with RFamide-labeled nerve cells. Sensory cells were seen projecting to the surface of the ectoderm in the tentacles (Fig 15A).

DISCUSSION

In this study, RFamide positive labeling was observed in neuronal elements of the *Hydra vulgaris* hypostome and tentacles. Anti-RFamide antibody labeled three distinct nerve ring structures as well as neurons and neurites throughout the ectoderm of the hypostome and tentacles.

This study provides the first immunohistochemical evidence for the existence of RFamide positive nerve rings in *H. vulgaris*, two of which appear to also be tubulin-containing nerve rings. One was coincident with a $GABA_B$ receptor labeled nerve ring (Hufnagel and Kass-Simon, manuscript in preparation). The existence of nerve rings in cnidarians may represent the first integration center of nervous tissues in the animal kingdom (Bullock and Horridge 1965). As described below, the nerve rings seen in this study were situated in areas of the hydra that have been proposed to coordinate specific behaviors.

A nerve ring was identified in the apical region of the hypostome and appeared to be composed of neurites emanating from sensory cells. The location and composition of this ring is consistent with a distal ring previously observed using anti-α-tubulin antibodies (Hufnagel and Kass-Simon, unpublished data) and also observed in our comparison preparations with anti-α-tubulin. As suggested by Hufnagel and Kass-Simon, due to the location and sensory nature of the ring, it is likely this ring plays a role in the feeding behavior of hydra.

A second ring, located directly below and between the tentacle attachment area was also identified in this study with RFamide antibody. This ring appeared to be

made up of bipolar nerve cells with long neurites. Stenoteles were observed to be intimately associated with this ring. A nerve ring in a similar location, also associated with clusters of stenotele nematocytes was observed by Hufnagel and Kass-Simon (manuscript in preparation), in preparations labeled with anti-α-tubulin antibodies (coincident with a ring that labeled with anti-$GABA_B$ receptor antibodies (Hufnagel and Kass-Simon, unpublished data)). The tubulin ring was reported to include neurites from sensory cell bodies located between tentacles. Due to their location, it is likely that the sensory cells contributing to this ring contain GABA or RFamide or both. In this regard it should be noted that in mammalian and other systems neurons have been found to contain both peptidergic and non-peptidergic neurotransmitters (Oertel 1983). The location of the proposed ring coincides with evidence from electrophysiological studies that suggests an ectodermal pacemaker system exists at or near the base of the tentacles (Kass-Simon 1972, 1973; Kass-Simon and Passano 1978) and is responsible for the periodic ectodermal body-column contractions. It has been shown that cnidarians require coordinated movements of their tentacles for feeding behaviors (Lenhoff 1961, Rushforth 1972; Westfall and Kinnamon 1984). Mechanical and chemical stimuli produced by prey swimming near the hydra, causes the discharge of nematocysts in the tentacles. Thus, poisoning and capturing the prey, which leads to the release of reduced glutathione (GSH) from the prey causing the tentacles to bend and bring the food to the mouth. The mouth opens, ingests the prey and closes (Lenhoff 1961).

The third ring structure observed was the circumtentacular nerve ring which encircled the base of each tentacle. These rings labeled with both anti-RFamide

antibody and anti-α-tubulin antibody. This is the first study to reveal such rings in H. *vulgaris*. However, other researchers have shown similar dense areas of nerves in immunochemical experiments with anti-α-tubulin (Hufnagel and Kass-Simon, personal communication) and similar rings have been described in the cnidarian *Chiropsalmus* by Anderson et al. (2004). Our finding that hydra's circumtentacular rings contain RFamide strengthens Golubovic's hypothesis that Hydra-RFamide contributes to the control of tentacular contractions, possibly during feeding (Golubovic et al 2007). A peptide-gated ion channel, the hydra sodium channel (HyNaC), was found in Hydra to be directly gated by Hydra-RFamides. In-situ hybridization methods were used to determine that cells expressing HyNaC were localized at the bases of the tentacles, adjacent to the neurons producing Hydra-RFamides. Results from electrophysiology experiments suggested that RFamides directly activate HyNaC by binding to the channel (Golubovic et al. 2007). Further research will be needed to determine the exact cell types of the ring observed in our study, which will also provide insight into the function these rings serve.

In addition to the nerve ring structures, RFamide immunoreactive neurons were observed elsewhere in the hypostome and tentacles. Especially apparent was the strong abundance of RFamide-positive sensory cells around the mouth, consistent with findings of others in *H. vulgaris* (Grimmelikhuijzen 1985). The abundance of sensory cells became more diffuse the farther away from the mouth. Neurites throughout the hypostome and tentacles showed punctate RFamide-labeling. Sensory cell bodies could be identified projecting to the surface of the hypostome around the mouth and in the tentacles. In the basal region of the hypostome, nests of developing nerve and

nematocyte cells (called neuroblasts and nematoblasts respectively) labeled with anti-RFamide antibody. Many of the developing cells look like nematoblasts due to the developing cysts but early stage nematoblasts and neuroblasts cannot be excluded. Neurons and nematocytes are continuously produced in the body column and then travel towards the apical end (hypostome or tentacles) or towards the basal end (basal disk) of the hydra (Bode et al. 1986; Bode 1992). Interestingly, research suggests that nematocyte and neuronal differentiation pathways share a common bipotent progenitor (Holstein and David 1990; Miljkovic-Licina et al. 2007); thus they can be considered sister cells (Galliot et al. 2009).

Nematocytes (cnidocytes), which are a distinguishing feature of cnidarians, are specialized cells that are used for a variety of functions including food capture, locomotion and defense against predators (Kass-Simon and Scappaticci 2002). Studies indicate that classical transmitters are involved in controlling nematocyte discharge and feeding. Physiological experiments found that glutamate, acting on NMDA receptors, and GABA, acting on $GABA_B$ receptors were involved in controlling cnidocyst discharge in ablated tentacles of *Hydra vulgaris* (Scappaticci and Kass-Simon 2008). In the hydrozoan, Corynidae dopamine was shown to be involved in modulating the discharge of nematocytes (Thurm et al. 1998).

In our experiments, nerve cells were readily seen to be associated with nematocytes in the basal part of the hypostome and predominantly in the tentacles. Within the tentacles, RFamide positive nerve fibers were observed extensively surrounding the nematocytes in specialized epitheliomuscular cells called battery cell complexes (Hufnagel et al. 1985). Most apparent and frequently observed were the

associations with the large, toxin-containing, stenotele nematocyte. Desmoneme nematocytes, which are much smaller than stenoteles were also observed to be associated with RFamide-positive nerve fibers. Another type of nematocyte, the isorhiza, is more difficult to identify so we were not able to distinguish if they were associated with RFamide neurons. These findings, which are supported in other classes of cnidaria, indicate at least part of the chemical coordinating system of the cnidaria is peptidergic. More specifically, Anderson et al (2004) described a distinct association of nematocytes and RFamide-immunoreactive neurons in the tentacles of *Chrysaora quinquecirrha* (Class Scyphoza), *Physalia physalis* (Class Hydrozoa), *Porpita porpita* (Class Hydrozoa) and *Chiropsalmus* (Class Cubozoa). The present finding of RFamide immunoreactive neurons associated with nematocytes provides the first clue that a neuromodulator may be involved in nematocyst function (Anderson 1992). Therefore, both the physiological and anatomical data suggest that the chemical control of hydra's behavior is not limited to one category of biochemical substance but that both classical neurotransmitters and neuropeptides are involved, especially in nematocyst discharge.

In summary, the present study indicates that the neurons of the hypostome and tentacle, particularly those that may be involved in coordinating feeding, nematocyst discharge and contractile behavior, contain RFamide, which is likely to participate in controlling these behaviors (Westfall and Kinnamon 1984).

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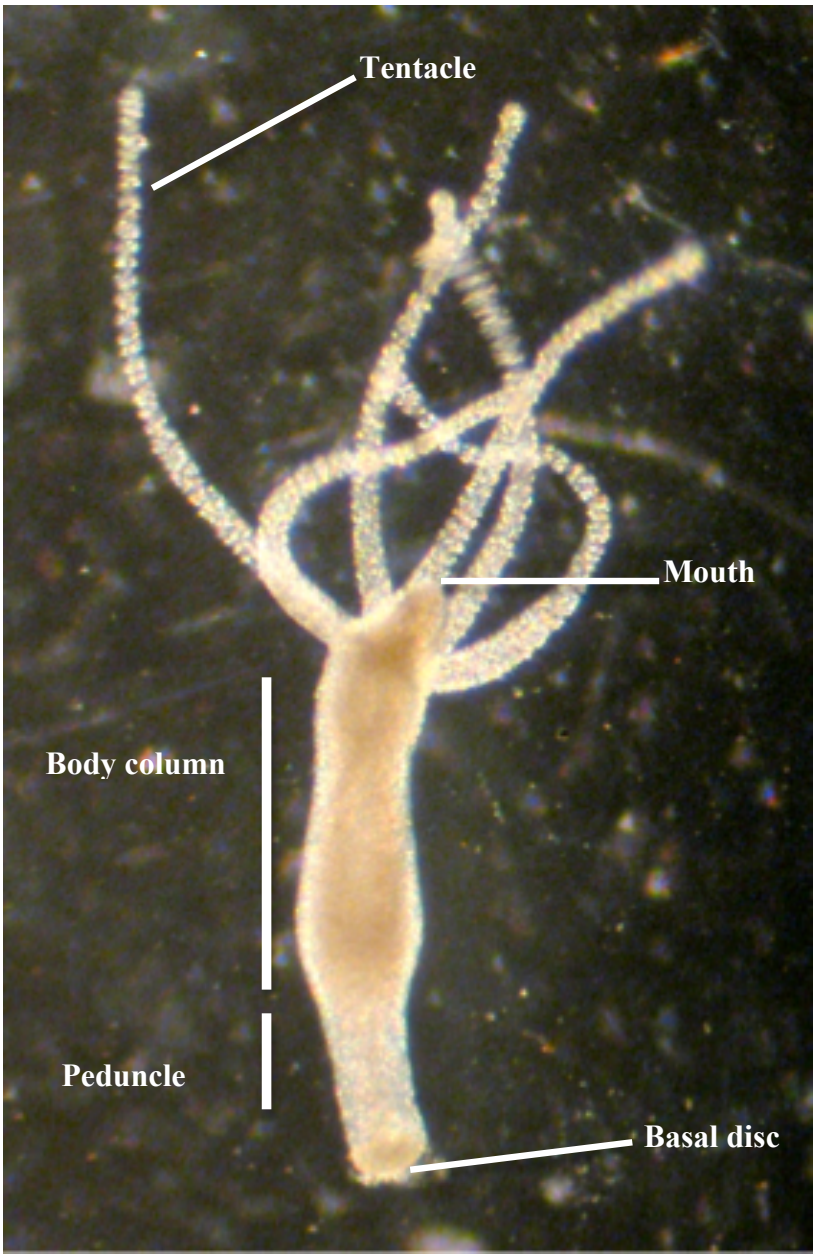

Figure 1. Image of *Hydra vulgaris* showing topographical organization (courtesy of Dr. Stephanie Guertin).

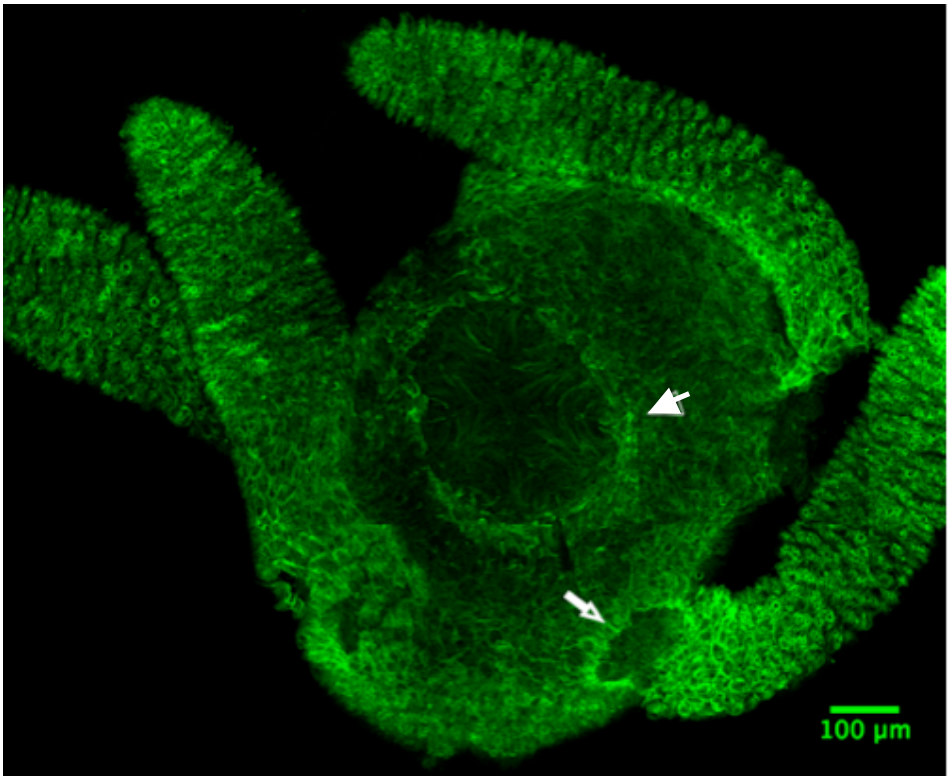

Figure 2. Scanning confocal image showing a circumferential nerve ring around mouth of hydra (arrow head) and at the insertion of a tentacles(arrow). Anti-α-tubulin. Size bar= 100 µm

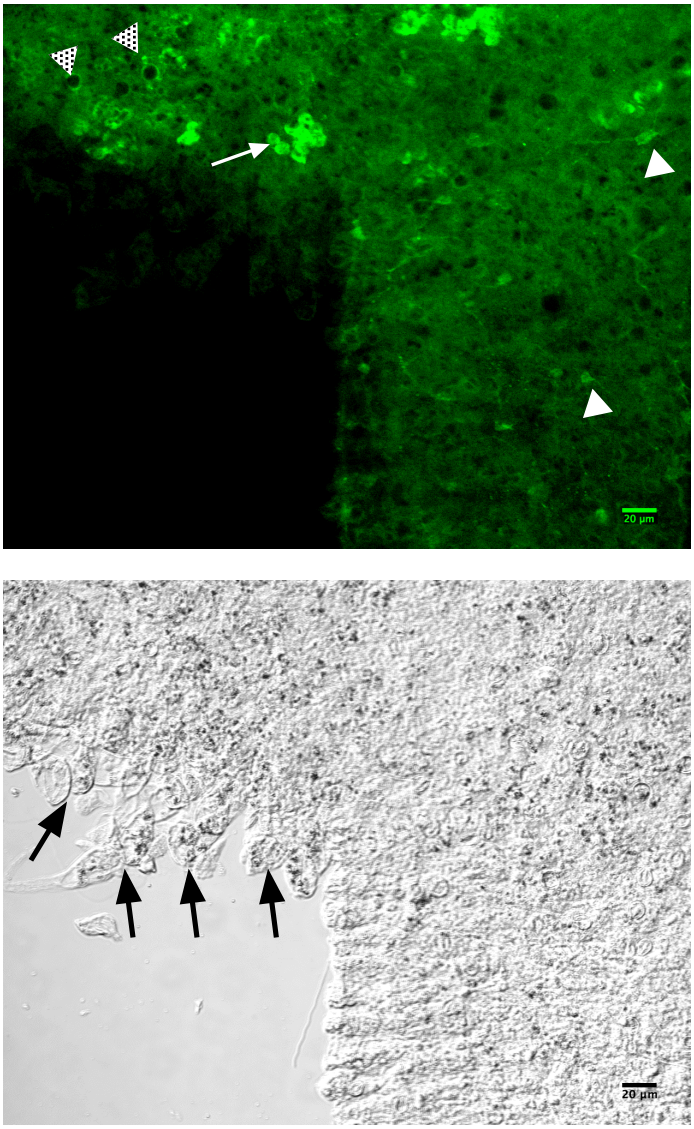

Figure 3. RFamide-positive labeling was cell-type specific. Anti-RFamide monoclonal antibody, labeled with Alexa488 fluorescent tag.

A. Labeling is seen in neurons (arrowheads), nematoblasts (arrow) and associated with nematocytes (dotted arrowheads) but not seen in epithelial cells. Size bar= 20 µm

B. Epithelial cells are seen in corresponding DIC image (arrow). Size bar= 20 µm

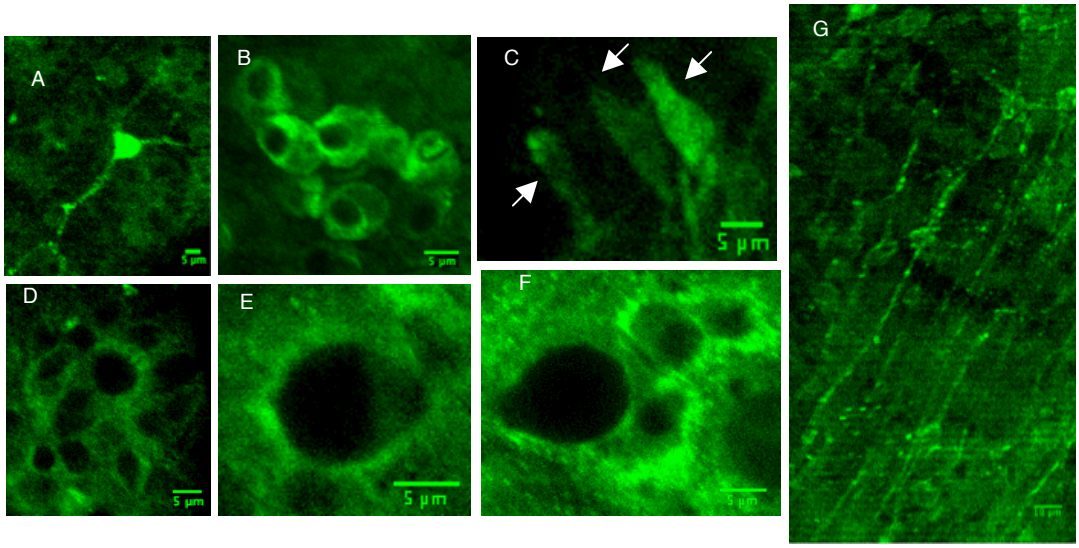

Figure 4. Examples of RFamide labeled neuronal structures.

A. Multipolar nerve cell. Size bar= 5 µm

B. Nest of developing nematoblasts. Size bar= 5 µm

C. Sensory cells (arrows). Size bar= 5 µm

D. Battery cell complex. Nematocysts do not label and appear as black circles and ovals. Size bar= 5 µm

E. Stenotele closely associated with RFamide positive nerve fiber. Size bar= 5µm

F. Stenotele and possibly three desmonemes innervated by RFamide positive nerves. Size bar= 5 µm

G. Neurites in the basal area of the tentacle. Size bar= 10 µm

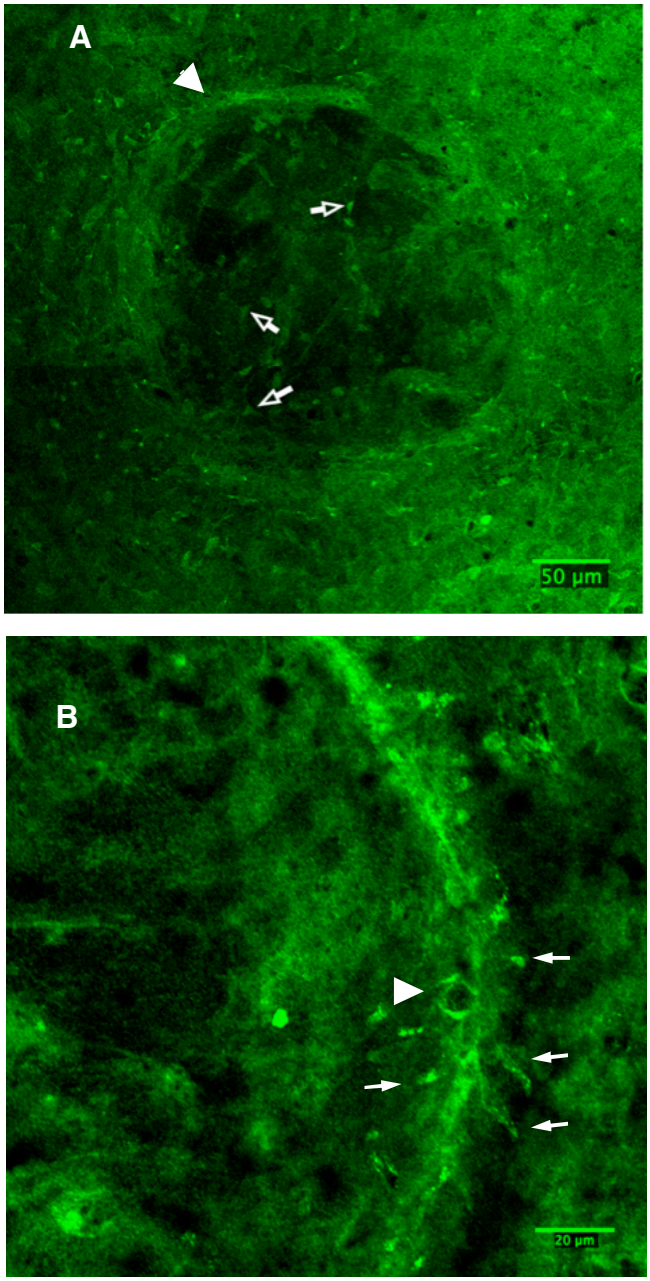

Figure 5. Hypostomes labeled with anti-RFamide antibody.

A. Fibers and cell bodies of a nerve ring (arrowhead) label with RFamide antibodies. Several cell bodies and radial fibers also label with RFamide (arrows). Size bar= 50 µm

B. A portion of the nerve ring. Sensory cells (white arrows) and nematocytes (arrowhead) are associated with the nerve ring. Size bar= 20 µm

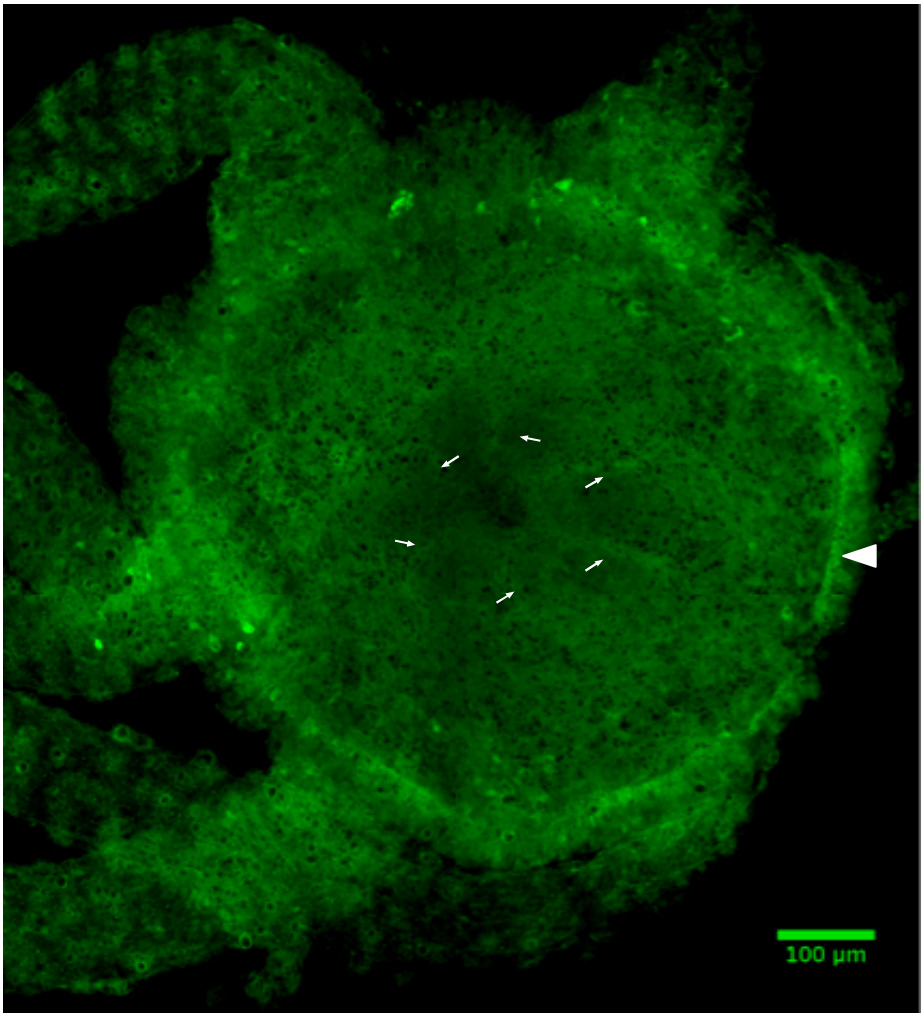

Figure 6. Horizontal optical section through the hypostome and tentacles at the level of the tentacle insertions. A proximally located nerve ring (arrowhead) is essentially complete and is found beneath the insertions of the tentacles. Note the six labial folds that form the mouth (arrows). Size bar= 100 µm. Anti-RFamide antibody, Alexa488 dye.

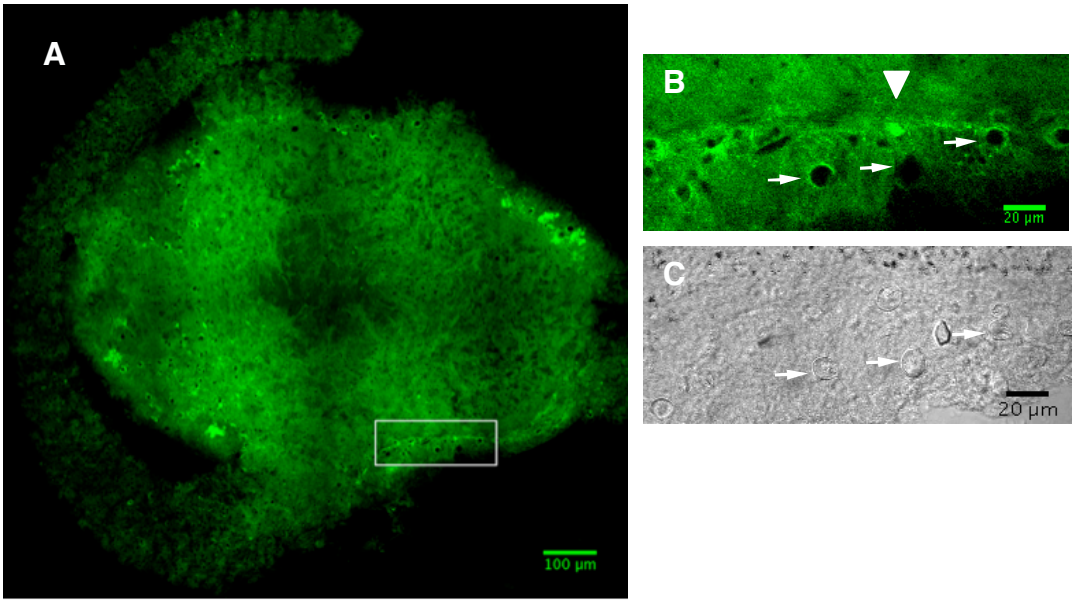

Figure 7. A RFamide-positive ring composed of labeled neuronal cell bodies and neurites as well as associated nematocytes.

A. White box indicates an intertentacular portion of ring. Size bar= 100 µm

B. Enlargement of boxed area in A, bright nerve cell body (arrowhead) and neurites with punctate labeling contribute to proximal nerve ring. Also note the stenoteles associated with the ring (arrows). Size bar= 20 µm

C. Corresponding DIC image. Size bar= 20 µm

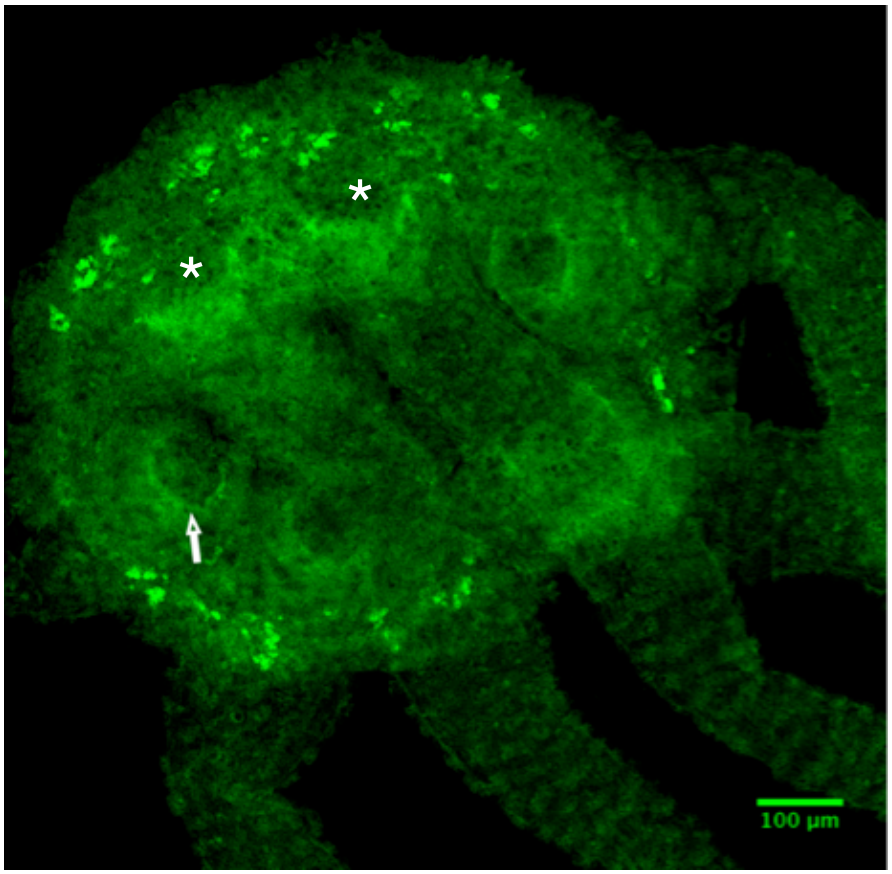

Figure 8. Horizontal optical section of the hypostome and tentacles viewed from beneath the tentacles, two of the tentacles (asterisks) are flipped onto and lie across the hypostome. A circumtentacular nerve ring is seen encircling the base of each tentacle (arrow). Size bar= 100 µm

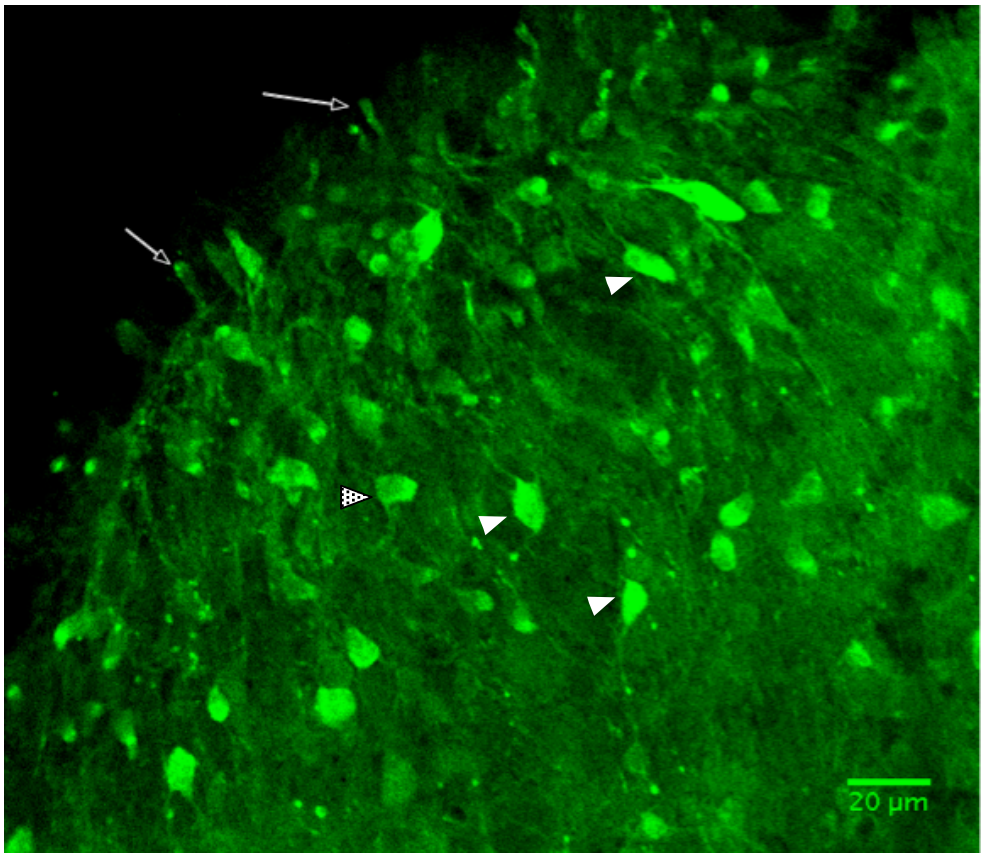

Figure 9. Tangential optical section through the ectoderm of the hypostome near the apex showing the strong abundance of anti-RFamide labeled nerve cells. Some sensory cells project to the surface of ectoderm (arrows). Note multipolar and bipolar cells (arrowheads). Labeling appeared to be distributed unevenly (dotted arrowhead). Size bar= 20 µm

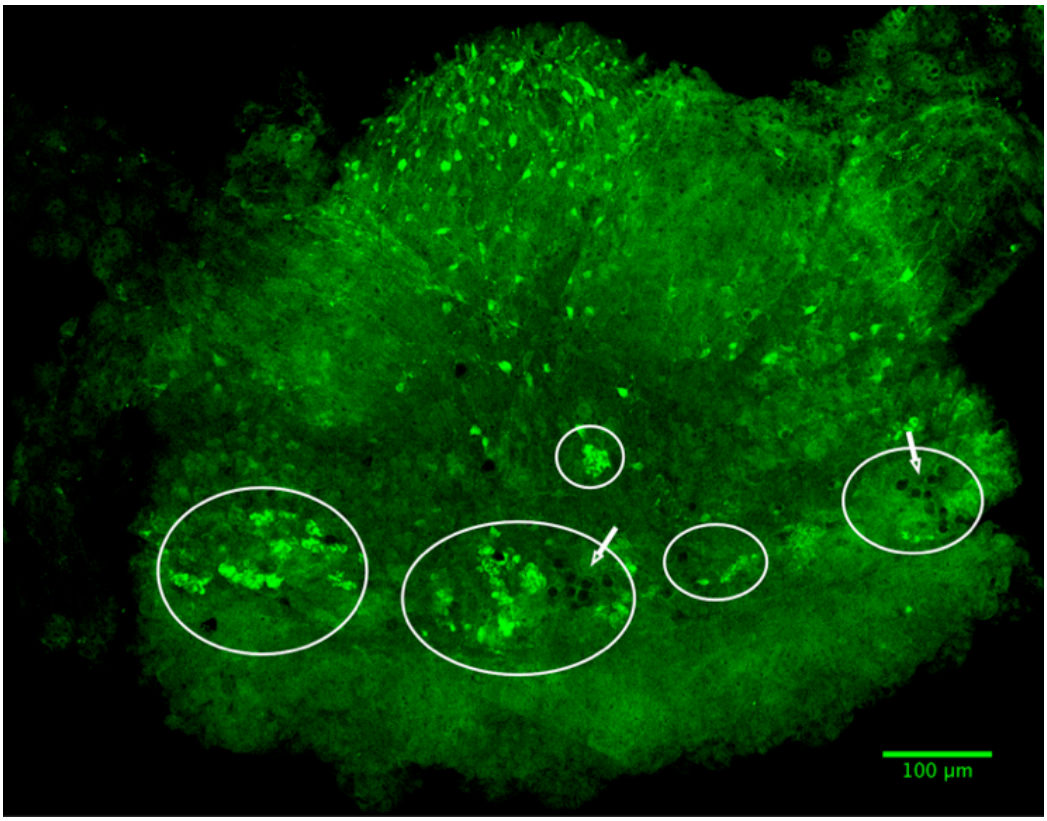

Figure 10. Tangential optical section through the hypostome and bases of tentacles. Clusters of nematoblasts are circled. They are located in the basal region of the hypostome (below tentacle attachment area). Arrows point to clusters of developing stenoteles. Size bar= 100 µm

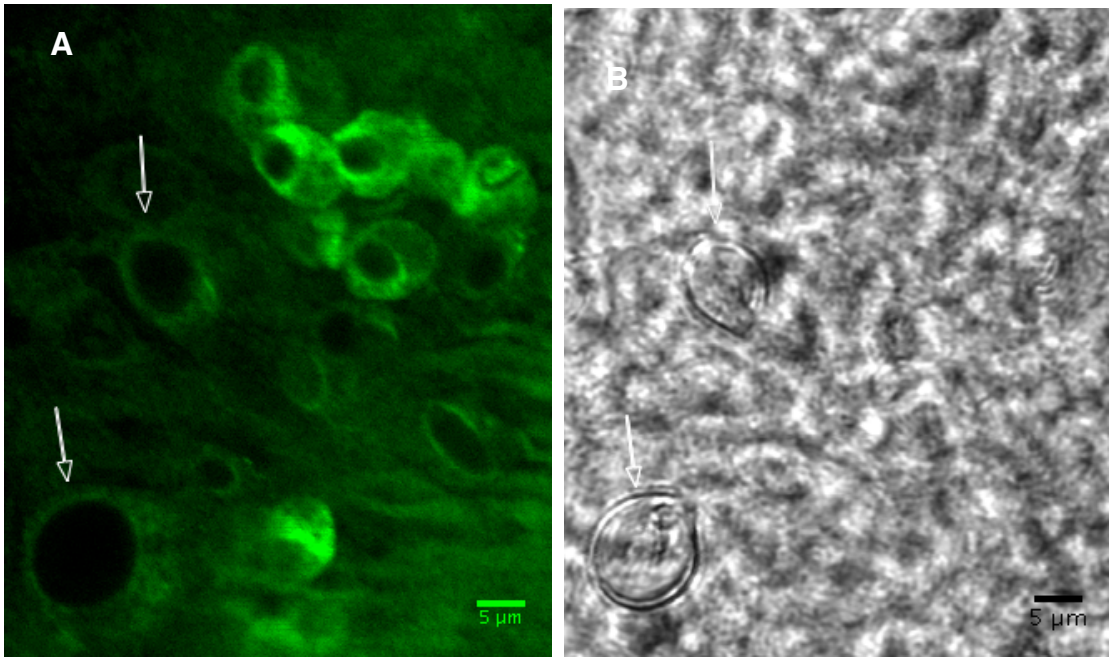

Figure 11. Cluster of nematoblasts in hypostome near tentacle attachment area.

A. Cluster of nematoblasts label brightly. Stenoteles (arrows). Size bar= 5 µm

B. Corresponding DIC image. Size bar= 5 µm

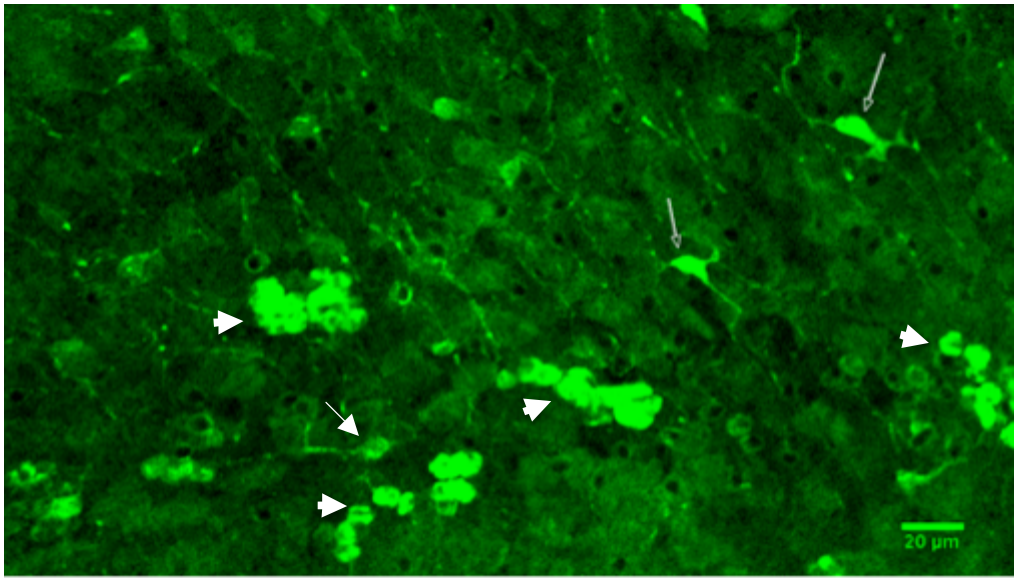

Figure 12. Basal region of hypostome, near tentacle attachment area. Brightly labeled tripolar nerve cells (arrows). Punctate labeling of neurites is evident. Nests of nematoblasts label brightly (arrowheads). Size bar= 20 µm

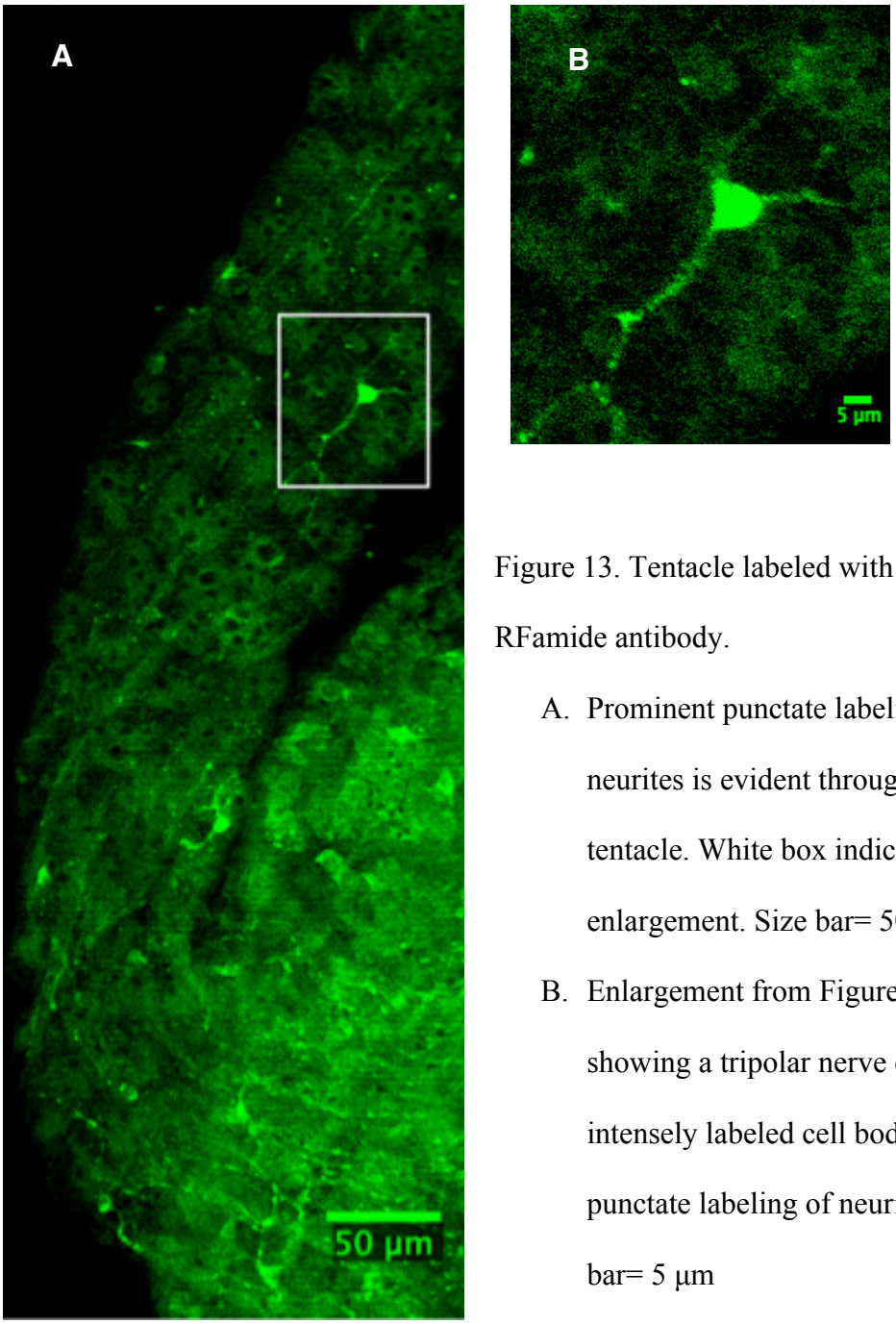

Figure 13. Tentacle labeled with anti-RFamide antibody.

A. Prominent punctate labeling of neurites is evident throughout entire tentacle. White box indicates enlargement. Size bar= 50 µm
B. Enlargement from Figure 12A, showing a tripolar nerve cell with intensely labeled cell body and punctate labeling of neurites. Size bar= 5 µm

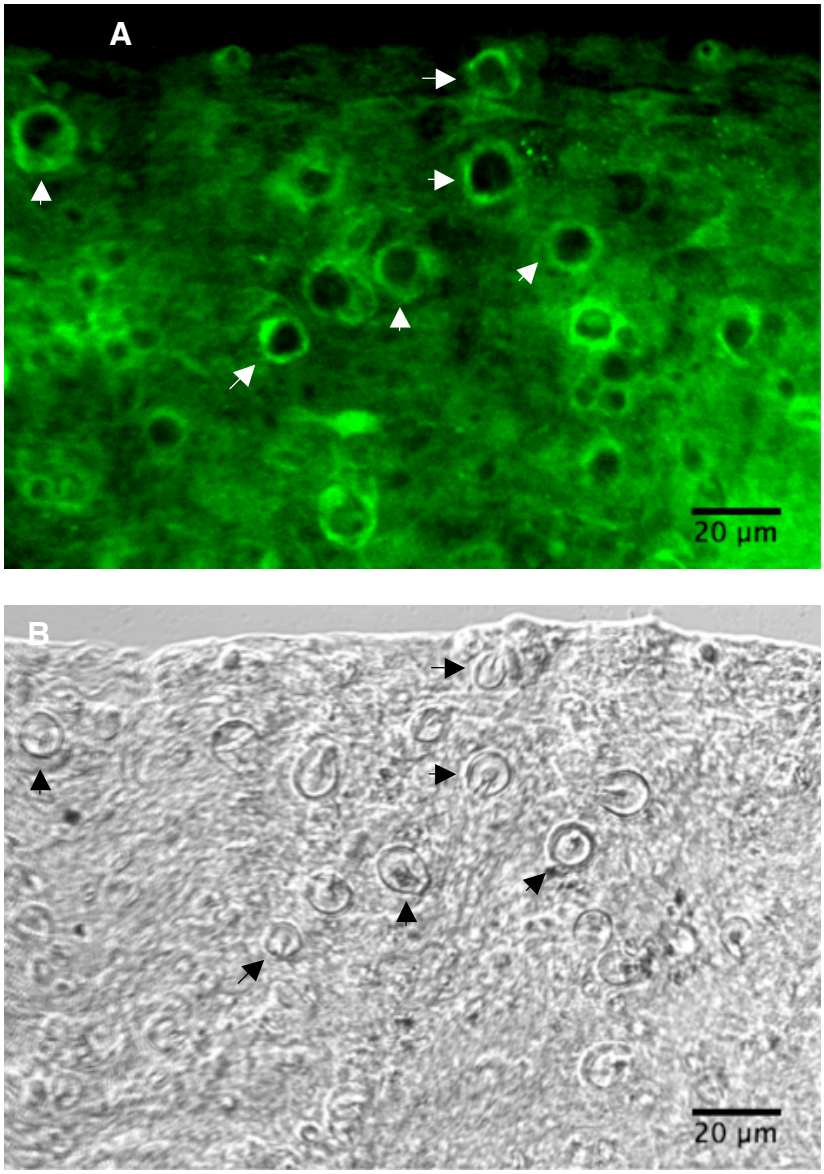

Figure 14. RFamide-positive label associated with nematocytes in the basal region of a tentacle.

A. Many stenotele nematocytes (arrows) are surrounded by rings of fluorescent label. The stenotele cysts appear as large black disks, while smaller disks are cysts of desmoneme nematocytes. Size bar= 20 µm

B. Corresponding DIC image. Several stenotele cysts are marked with black arrows. Size bar= 20 µm

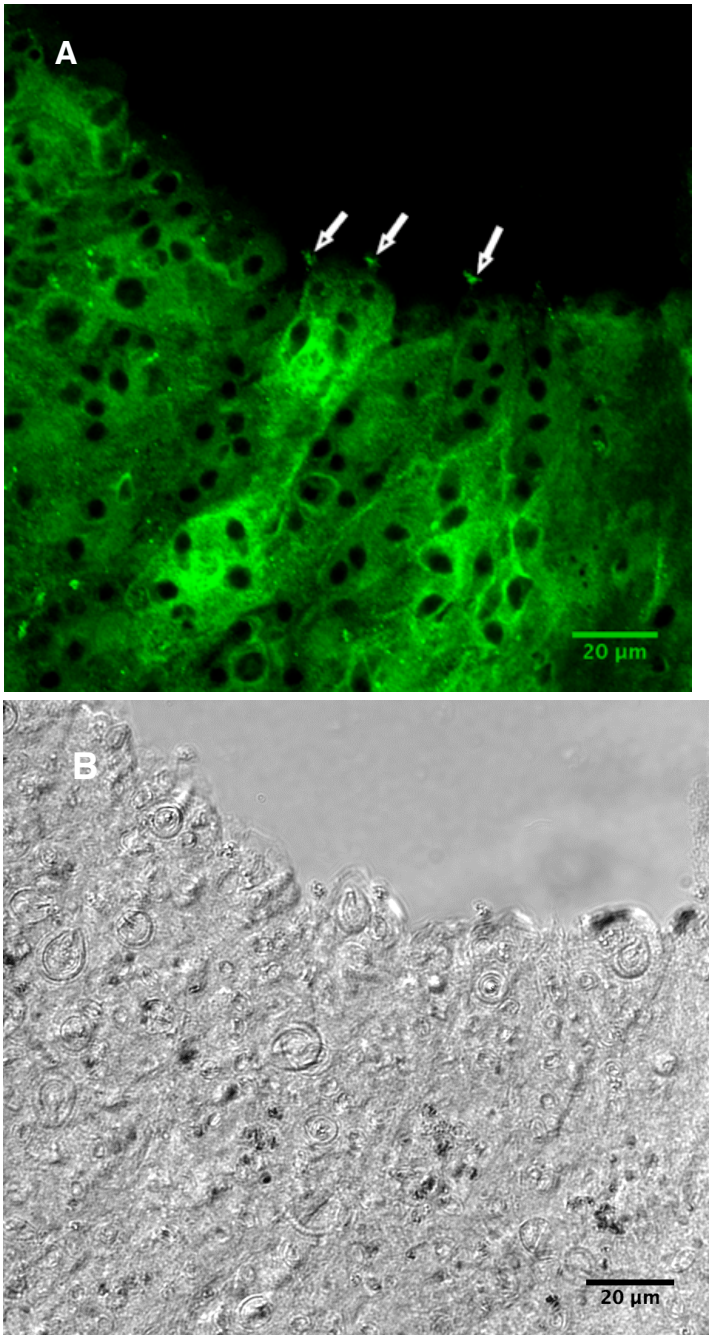

Figure 15. RFamide-positive neurites associated with nematocytes in battery cell complexes of the basal region of a tentacle.

A. Distinct punctate labeling of neurites that are abundant around the nematocytes in battery cell of tentacle. Patches of label are also found on sensory cells that project to surface (arrows). Size bar= 20 µm

B. Corresponding DIC image. Size bar= 20 µm

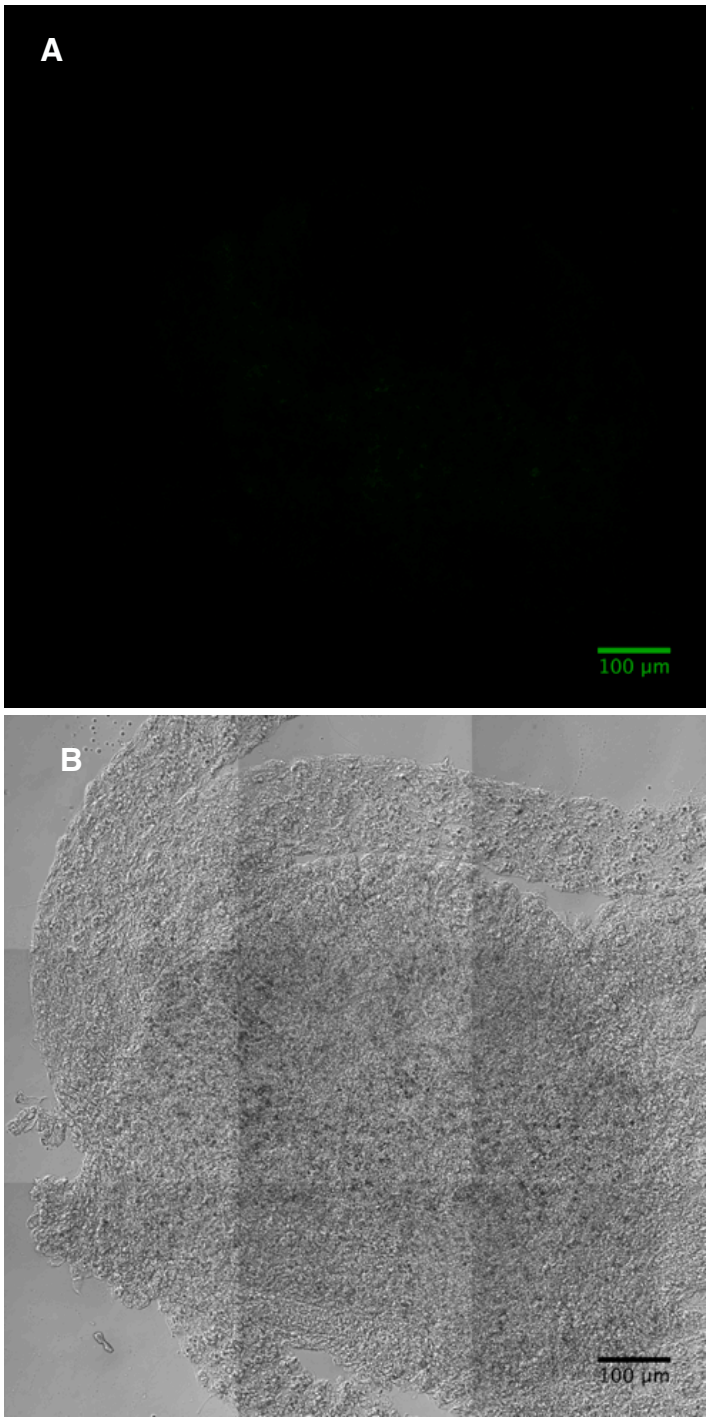

Figure 16. Negative control slide- primary antibody was omitted. Hypostome and tentacles.

A. Fluorescence image Size bar= 100 µm

B. Corresponding DIC image. Size bar= 100 µm

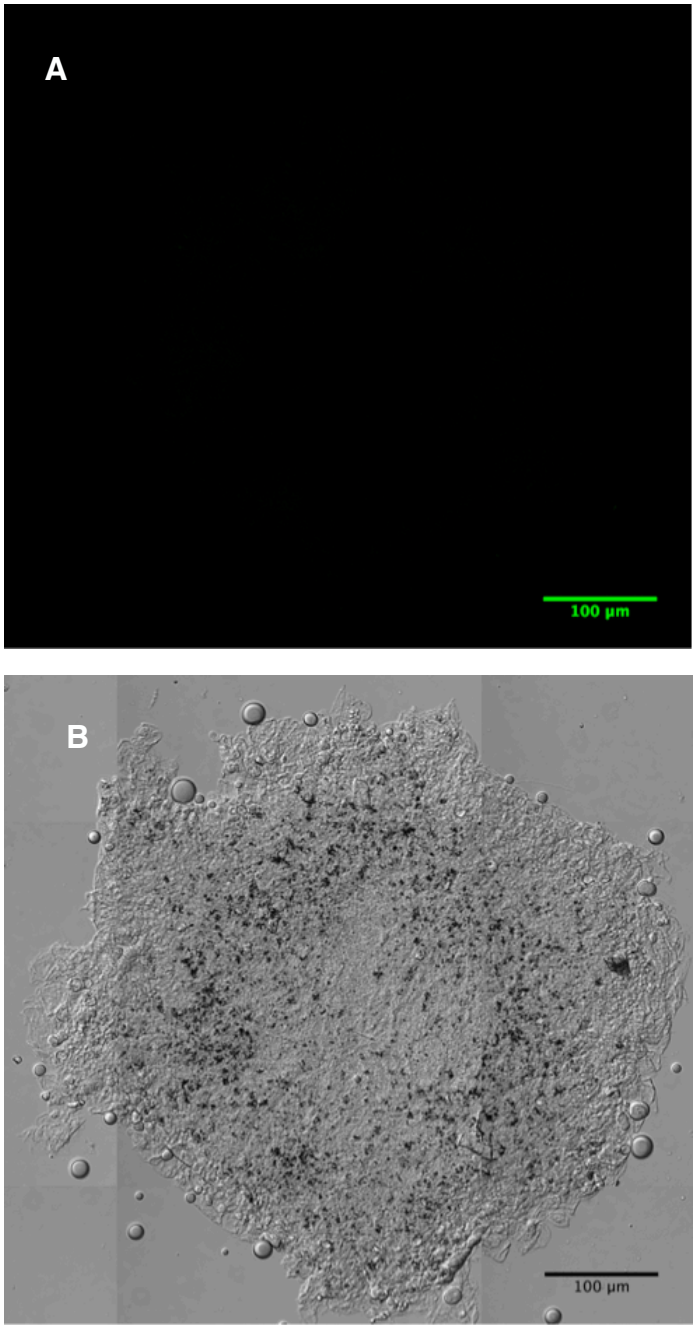

Figure 17. Negative control slide- primary antibody was omitted. Hypostome with no tentacles attached.

A. Fluorescence image. Size bar= 100 µm

B. Corresponding DIC image. Size bar= 100 µm

APPENDICES

I. BACKGROUND

A. General RFamide

Neuropeptides that terminate in arginine-phenylalanine-amide (RFamide) were first isolated from the ganglia of the clam *Macrocallista nimbosa* and reported to exert cardioexcitatory effects (Price and Greenberg 1977). Since then the RFamide neuropeptide family has been shown to have important roles in the nervous system of both vertebrates and invertebrates (Darmer et al. 1991). It appears that the genes encoding these neuropeptides emerged early in evolution and have been relatively well conserved (Dockray 2004). In mammals, five RFamide peptide genes have been identified as well as at least five G-protein-coupled receptors on which the peptides act (Findeisen et al. 2011).

Several different neuroreactive peptides are encoded in a single continuous mRNA, which is translated into one large preprohormone (also referred to as a polyprotein) (Kandel 2000). Neuropeptides are synthesized from the preprohormones, which are large precursor proteins that consist of an N-terminal signal sequence and a prohormone part containing one or more copies of the immature peptide (Grimmelikhuijzen et al. 1996). The signal sequence is cleaved off during translocation into the endoplasmic reticulum and the prohormone part is subsequently transported into the Golgi system, where it is sorted and packaged into neurosecretory vesicles. Several processing enzymes convert the prohormones into their biologically active peptide (Grimmelikhuijzen et al. 1996).

RFamide neuropeptides have been shown to be involved in a variety of behaviors. A growing body of evidence suggests the peptides play a role in the feeding behavior of a wide range of species, including mammals, coelenterates (Mackie et al. 2003), nematodes (de Bono and Bargmann 1988) and mollusks (Sossin et al. 1987). RFamide neuropeptides were found widely distributed in the rat central nervous system with possible roles in the neuroendocrine system and pain modulation (Yano et al. 2003). RFamide neuropeptides have also been demonstrated to have a cardioregulatory function in humans (Shimizu and Fujisawa 2003).

B. Cnidarian RFamide

Cnidaria consists of four classes, Anthozoa, Scyphozoa, Cubozoa and Hydrozoa (includes hydra). RFamide has been identified in all of them. Antisera against the RFamide sequence demonstrated for the first time the presence of a peptidergic neuronal centralization in *Hyda* (Grimmelikhuijzen 1985). Nearly a decade later, the structure of RFamides from hydra were determined using radioimmunassays. Four peptides were found to all have an RFamide C terminus: Hydra-RFamide I-IV (Moosler et al. 1996). Hydra RFamides were identified to function in the induction and inhibition of muscle contractions; in particular, Hydra RFamides III and IV were identified to enhance "body pumping" (Fujisawa et al. 2012). More specifically, it was demonstrated using India ink solution that when *Hydra* are elongated, their peduncle (lower quarter of the body column) stores much of the gastrovascular fluid; when contracted, the peduncle uses pumping movements to transfer that fluid into the entire gastric cavity. RFamide was shown to elevate this pumping action (Shimizu and Fujisawa 2003). *Hydra* has three preprohormones which give rise to RFamides I-IV. Preprohormone A contains copies of all four RFamides, preprohormone-B contains copies of RFamide I and II and two putative RFamide neuropeptide sequences and preprohormone-C contains one copy of RFamide I and seven copies of other putative neuropeptide sequences (Darmer et al. 1998).

It has been demonstrated that neuropeptides are distributed within neurites and in cell bodies, supporting the hypothesis that they play a role in neurotransmission (Grimmelikhuijzen 1985, Koizumi et al 1989). It was suggested that a function of the RFamide peptide family is to regulate the myoactivity and/or modulation of muscle

contraction (McFarlane et al. 1987; McFarlane et al. 1991). It has been shown using immuno-electromicroscopy that RFamide neuropeptides in *H. magnipapillata* are located in dense-cored neurosecretory vesicles in axon terminals that end on the myonemes of epitheliomuscular cells. This suggests a possible function of RFamide-like peptides in neuromuscular transmission in hydra (Koizumi et al 1989). Studies in sea anemones (class Anthozoa) have shown Antho-RFamide neuropeptides in neuronal dense-cored vesicles associated with two-way neuroneuronal synapses (Westfall and Grimmelikhuijzen 1993), which suggests the involvement of RFamide in neuroneuronal communication in sea anemones.

The genus *Hydra* includes four groups: common hydra, stalked hydra, green hydra and gracile hydra. Color, presence or absence of a body stalk, nematocyst shape, and the order of appearance of tentacles on the new buds are characteristics used to differentiate between the genus groups (Campbell 1987). The RFamide neuropeptide family has been extensively studied in the four groups, all of which contain RFamide positive nerve cells in the hypostome (Koizumi 2007). Yet, there has been varying morphological descriptions of the distributions and centralizations of these RFamide positive nerve cells between species. Until now, immunohistochemical studies with anti-RFamide antibodies have shown the presence of circumhypostomal nerve rings in *H. oligactis* (stalked), *H. robusta* (stalked) and *H. circumcincta* (gracile), but not in *H. vulgaris* (common), *H. viridissima* (green) or *H. magnipapillata* (common) (Grimmelikhuijzen 1985; Grimmelikhuijzen et al 1989; Takashi et al 2003; Koizumi et al 1992, 2004; Koizumi 2007). More precisely, an RFamide positive nerve ring was demonstrated in species categorized as stalked and gracile hydra (Koizumi 2007). In

*Hydra vulgaris (*common hydra) immunohistochemical studies using antibodies against RFamide previously had not identified a nerve ring, only that two neuronal centralizations occur, in the ectoderm of the hypostome and the lower peduncle (Grimmelikhuijzen 1985). Specifically, RFamide positive sensory neurons were located in the ectoderm of the hypostome, around the mouth opening (Grimmelikhuijzen 1985; Plickert 1989; Koizumi et al. 1992) and RFamide positive ganglion cells were located in the tentacles and peduncle as well as in the hypostome (Grimmelikhuijzen et al. 1991; Koizumi 2002).

Although immunohistochemical localization of RFamide has proven to be a valuable tool in visualizing the nervous system in cnidarians, it is possible that only a portion of the nervous system is visualized this way. It may be that not all neurons of cnidarians contain RFamides.

Other peptides, as well as classical neurotransmitters, have been shown to be involved in neurotransmission (Pierobon et al. 2001; Scappaticci et al. 2004; Kass-Simon and Pierobon 2007; Scappaticci and Kass-Simon 2008; Pierobon 2012). Including glutamate (Hufnagel and Kass-Simon 1995), $GABA_B$ (Hufnagel and Kass-Simon, MS in preparation), glycine (Pierobon et al. 2001) and acetylcholine (Kass-Simon and Pierobon 2007).

II. α-TUBULIN LABELED HYPOSTOME AND TENTACLE STRUCTURES

Experiments were also run on hydra hypostome and tentacles (*H. vulgaris*) using antibodies against α-tubulin, a protein that is abundant in nerve cell bodies and neurites. An anti-α-tubulin mouse monoclonal antibody (Sigma Clone B512), which has been shown to provide consistent and reliable results in *H. vulgaris*, was used for comparative purposes. Previous research incorporating this particular antibody indicated the presence of a proximal and distal nerve ring (Hufnagel and Kass-Simon, manuscript in preparation), and a driving question of this research was whether either or both of those nerve rings were also RFamide positive. In addition to corroboration of the previous finding of tubulin-associated hypostomal nerve rings in *H. vulgaris* it was also useful to use this antibody for trouble-shooting purposes. Since there are a number of variables that can affect immunochemical experiments (ex: fixation method, temperature, incubation period) and we were not experienced with the anti-RFamide antibody, it proved a valuable tool to be able to label some of the tissue samples with this antibody alongside samples labeled with the anti-RFamide antibody.

Methods used are described in more detail in the thesis. Briefly, individual hydra were transected directly underneath the tentacle/ hypostome region using a scalpel; the body was discarded leaving only the tentacle and hypostome. The remaining steps were done in a moist chamber and the preparations were washed with PBS and mPBS between steps. The tissue was treated with dissociation medium followed by Zamboni's fixative (Zamboni et al. 1967) and incubated in 0.4M glycine, primary antibody and secondary antibody tagged with Alexa488 fluorescent label.

Lastly, mounting medium and coverslips were applied and sealed with nail polish. Slides were evaluated using a Zeiss LSM700 confocal system equipped with ZENBlack imaging software.

Circumtentacular nerve rings were observed to label with the anti-α-tubulin mouse monoclonal (Fig. 1A and 1B). The rings, consistent with those seen in anti-RFamide preparations, were seen to encircle the base of the tentacles- this was especially clear in instances when the entire tentacle was missing from the hypostome. A great number of sensory cells appeared to label at the apex of the hypostome (Fig. 2A and 2B) in a similar location to that seen with anti-RFamide antibody.

Monoclonal antibodies are produced from a single B-cell clone from one animal, therefore they are immunochemically identical and react with a specific epitope of the antigen against which they were raised. Polyclonal antibodies are produced from different B-cell clones of an animal, in consequence, they are immunochemically dissimilar and react with various epitopes on the antigen against which they were raised (Naish et al. 1989). Both types of antibodies can be produced in various animal types but as of now monoclonal antibodies are commonly produced using mice and polyclonal antibodies are frequently produced using rabbits (Naish et al. 1989). It is important to know what animal the primary antibody is made in when applying a secondary antibody since they should be raised against the same species. When performing double immunostaining experiments using unconjugated primary antibodies it is necessary to use antibodies that are produced in different species and that the secondary antibody recognizes one of those species exclusively.

An initial goal of this project was to conduct double-labeling experiments, using the anti-RFamide monoclonal antibody we received from O. Koizumi and the anti-α-tubulin monoclonal antibody made in mouse on the same hydra but at the time we were not equipped to carry out that work. Since both antibodies were made in mice, we were faced with the issue of cross-reactivity between the two secondary antibodies directed toward the primary antibodies. In an effort to solve this problem, we tested an anti-α-tubulin monoclonal antibody made in rabbit (EMD Millipore clone EP13324). Unfortunately, initial trials of this antibody did not produce satisfactory results; this was consistent with previous researchers' findings (Hufnagel, personal communication). The antibody did not provide dependable results, labeling was faint and specific cellular structures were hardly identifiable; even when the brightness and contrast of the images were increased, the results were sub-par (Fig. 3). In one preparation, labeling with anti-α-tubulin rabbit monoclonal antibody was seen perhaps associated with the proximal ring (Fig. 4A) as well as with the circumtentacular rings (Fig. 4B). Unfortunately, these results could not be reproduced, the labeling of nerve cells and fibers were faint and sporadic and there was no clear sign of a nerve ring. Due to the inconsistency of the labeling, it was not possible to use the rabbit monoclonal antibody in double-labeling experiments.

Directly tagged with AlexaFluor488 anti-tubulin-α-mouse IgM (Biolegend) antibody was also tested. This anti-α-tubulin mouse antibody was already pre-labeled with AlexaFluor488, the hope was we would be able to use this antibody in double labeling experiments with the anti-RFamide mouse antibody. Unfortunately, it did not label the hypostome and tentacles adequately. Labeling was extremely faint, even

when brightness and contrast were digitally adjusted to be comparable to similar images the tissue was barely visible (Fig 5A and C). In order to see the tissue, the brightness and contrast were required to be increased to close to maximum (Fig. 5B and D). Similar to the rabbit antibody, the labeling was not consistent and varied even within samples from the same experiment. It was apparent that labeling with the two anti-α-tubulin antibodies, even at their best, was not comparable to the original anti-α-tubulin antibody made in mouse (Sigma Clone B512) when tested with *Hydra vulgaris*. In order to overcome these problems, in future studies, methods for directly labeling the anti-α-tubulin mouse monoclonal antibody (Sigma Clone B512) may be necessary. Such studies were planned but could not be carried out to limited time and resources.

III. GABA$_B$ LABELED HYPOSTOME AND TENTACLE STRUCTURES

Experiments were conducted on hydra hypostome and tentacles using anti-GABA$_B$ R1 rabbit polyclonal antibody (Chemicon AB5850), which is specific for the GABA$_B$ receptor 1 subunit, N-terminus. Previous research using this particular antibody revealed a labeled ring coincident with the anti-α-tubulin labeled proximal ring (Hufnagel and Kass-Simon, manuscript in preparation). In an effort to try to determine if the anti-RFamide proximal nerve ring was coincident with the anti-GABA$_B$ receptor nerve ring, samples were double-labeled with the two antibodies. Since the two antibodies were made in different host species (RFamide made in mouse, GABA$_B$R made in rabbit) we were able to carry out double labeling with the two primary antibodies. The dye Texas Red (wavelength 595nm) was used in the double-labeling experiments and unfortunately the Zeiss laser confocal microscope in the URI Genomics and Sequencing is not currently equipped with any filter cubes or lasers lines that excite at that wavelength. Therefore we were not yet able to examine and take digital images of the samples showing both the RFamide and GABA$_B$R labeling using the available confocal laser scanning microscope. When the GABA$_B$R labeling was able to be examined (due to it being labeled with a secondary antibody tagged with AlexaFluor488 dye) using the confocal microscope (meaning it was not possible to visualize the RFamide labeling), it was possible to identify structures similar to the circumtentacular nerve rings identified in separate samples with anti-RFamide and anti-α-tubulin antibodies. Further experiments will need to be done to

confirm if the three labeled rings are the same, it is nevertheless, very likely that the circumtentacular nerve rings have both peptidergic and GABAnergic properties.

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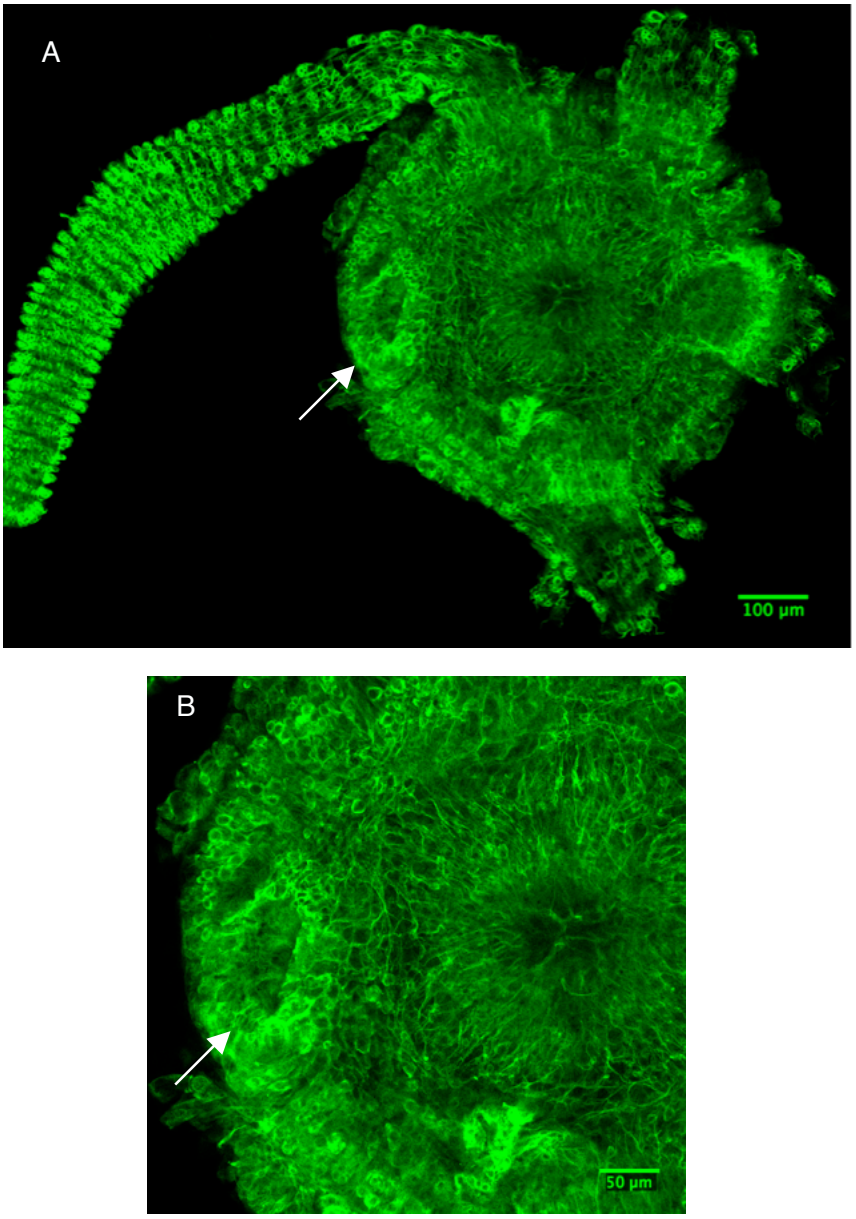

Figure 1. A hypostome/ tentacle piece labeled with anti-α-tubulin mouse monoclonal antibody.

A. Hypostome with one complete tentacle and four tentacle bases. Complete circumtentacular nerve ring (arrow). Size bar= 100 µm

B. Enlargement of a portion of Figure 1A. Circumtentacular nerve ring (arrow). Nerves from the hypostome connect with the ring. Size bar= 50 µm

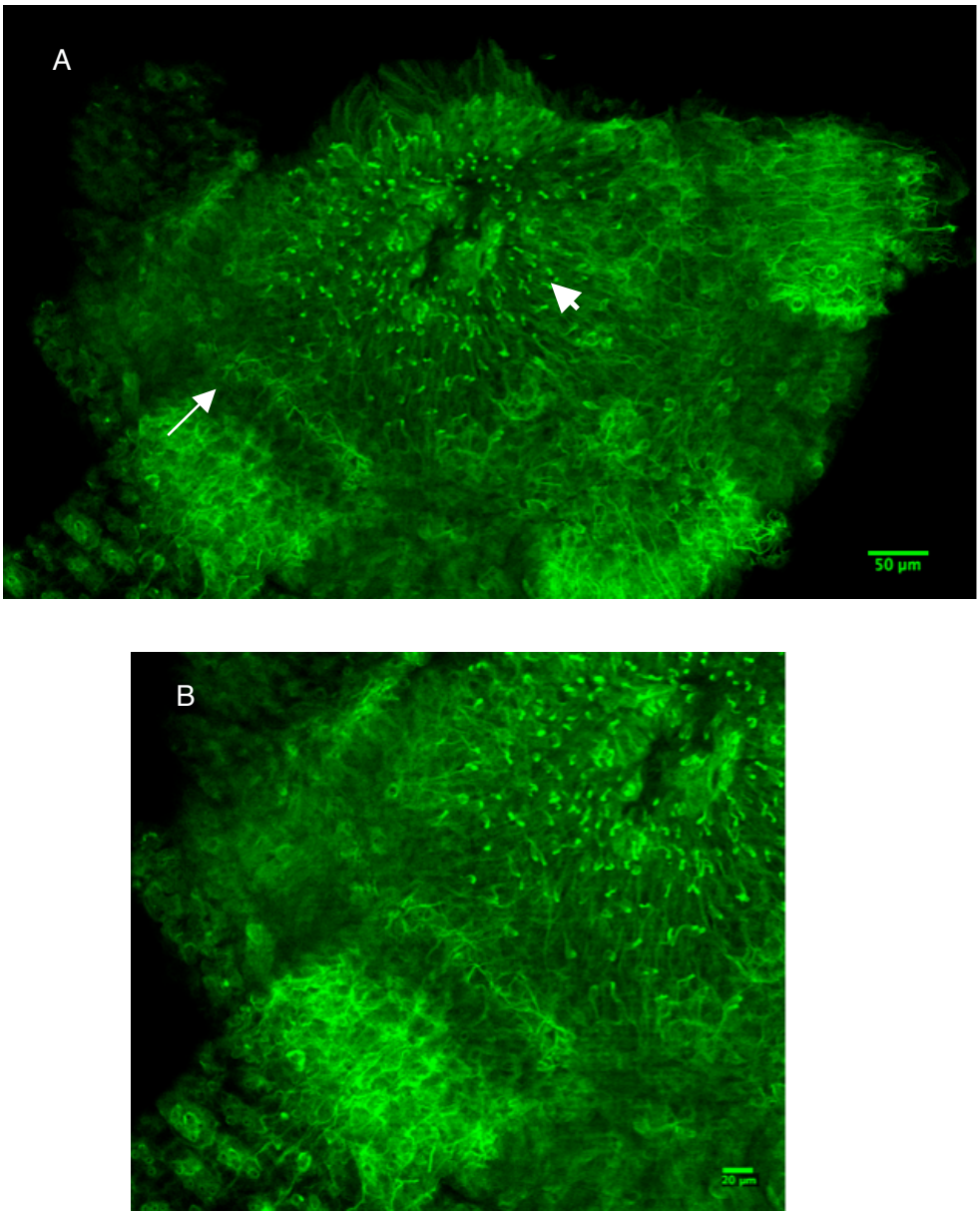

Figure 2. Scanning confocal section through a hypostome and bases of tentacles. Anti-α-tubulin mouse monoclonal antibody.

A. Sensory cells are visible around mouth (arrowhead). There is strong labeling at base of tentacles (arrow). Size bar= 50 µm

B. Enlargement of Figure 2A. Size bar= 20 µm

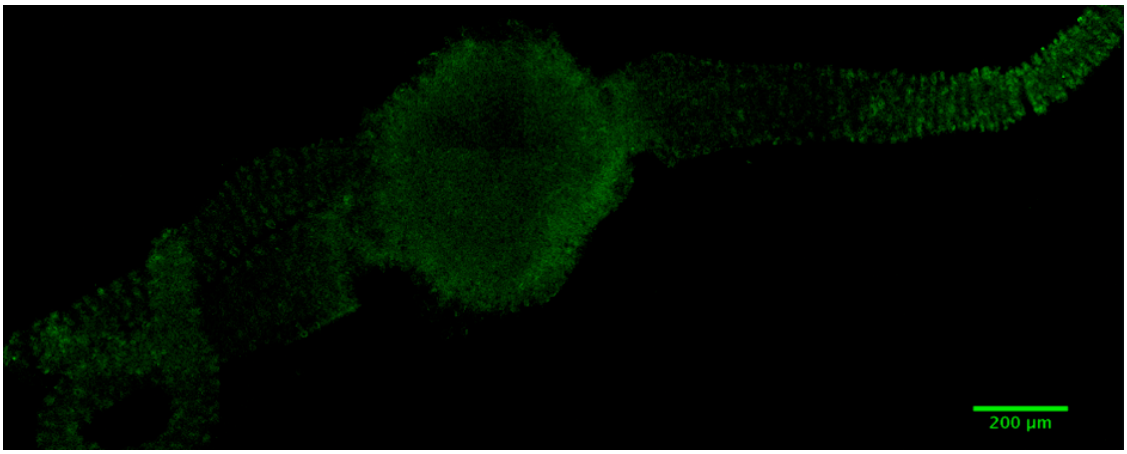

Figure 3. Confocal image of hydra hypostome and attached tentacles labeled with anti-α-tubulin rabbit monoclonal antibody showing faint labeling. Size bar= 200 µm

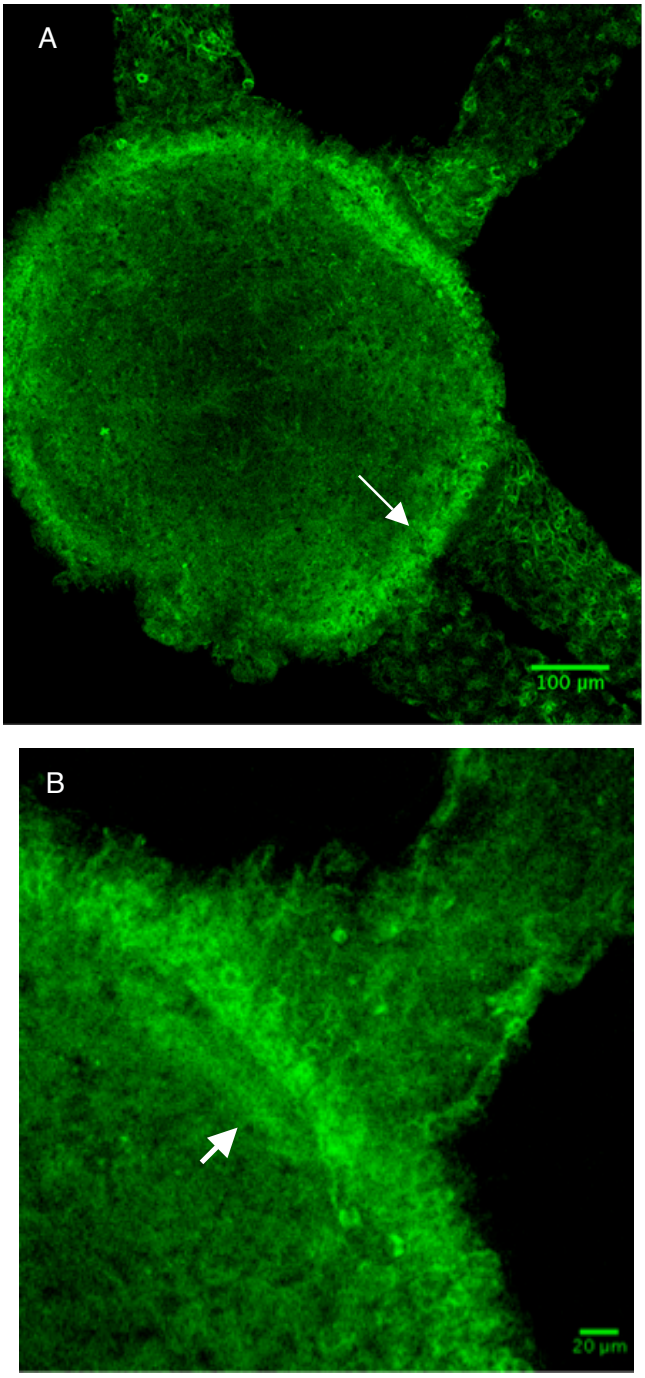

Figure 4. A hypostome/ tentacle labeled with anti-α-tubulin rabbit monoclonal antibody.

A. Concentrated labeling in outer hypostome proximal nerve ring (arrow). Size bar= 100 µm

B. Enlargement of Fig 4A. Circumtentacular nerve ring (arrowhead). Note: In comparison to anti-α-tubulin mouse monoclonal, this antibody labeling is not as extensive or bright. Size bar= 20 µm

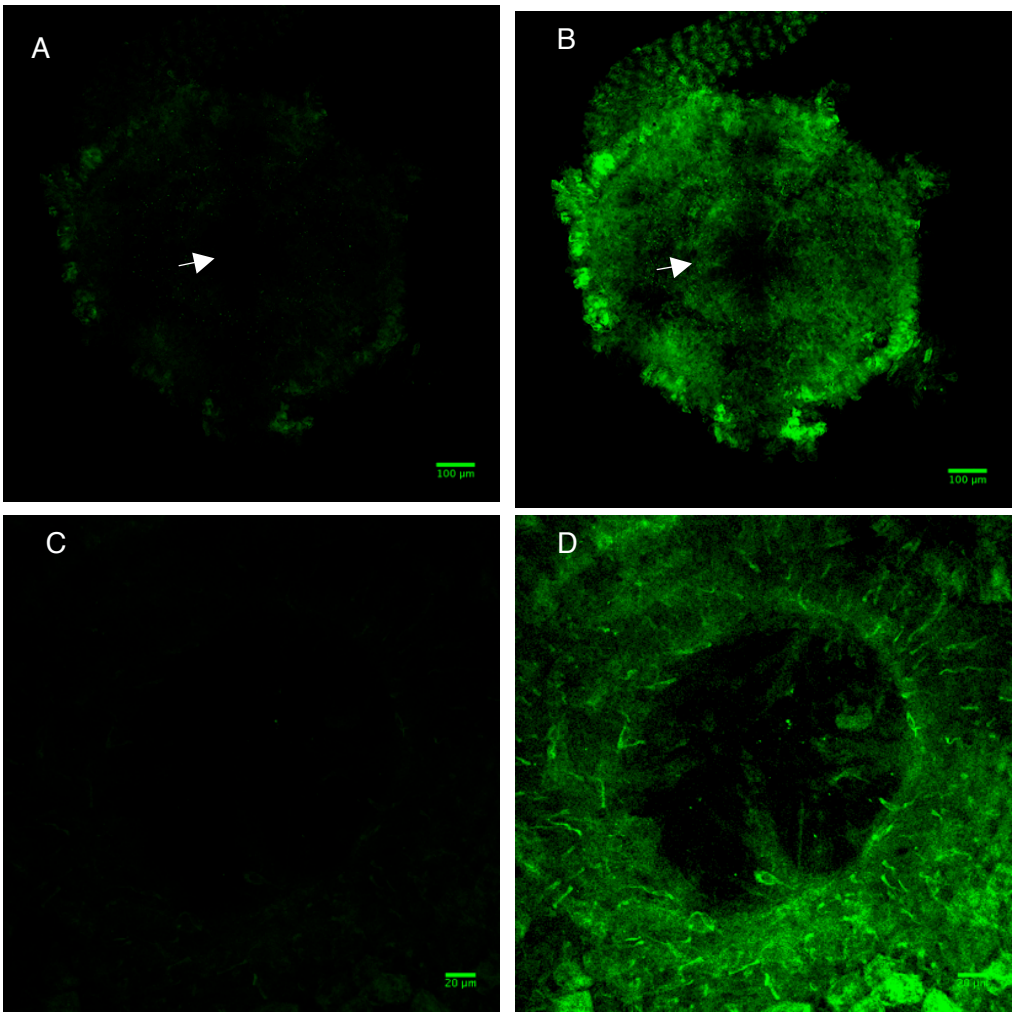

Figure 5. Confocal image of hypostome/ tentacle labeled with anti-tubulin-α-mouse IgM antibody directly tagged with AlexaFluor488.

A. Brightness and contrast adjusted comparable to that of other images (note the tissue is barely distinguishable due to faint labeling). Size bar= 100 µm

B. Image 5A with brightness and contrast increased to just below maximum. Arrows indicate the mouth area of the hypostome. Size bar= 100 µm

C. Image of mouth, brightness and contrast adjusted comparable to that of other images (note the tissue is barely distinguishable due to faint labeling). Size bar= 20 µm

D. Image 5C with brightness and contrast increased to just below maximum. Sensory cells are now distinguishable around the mouth. Size bar= 20 µm

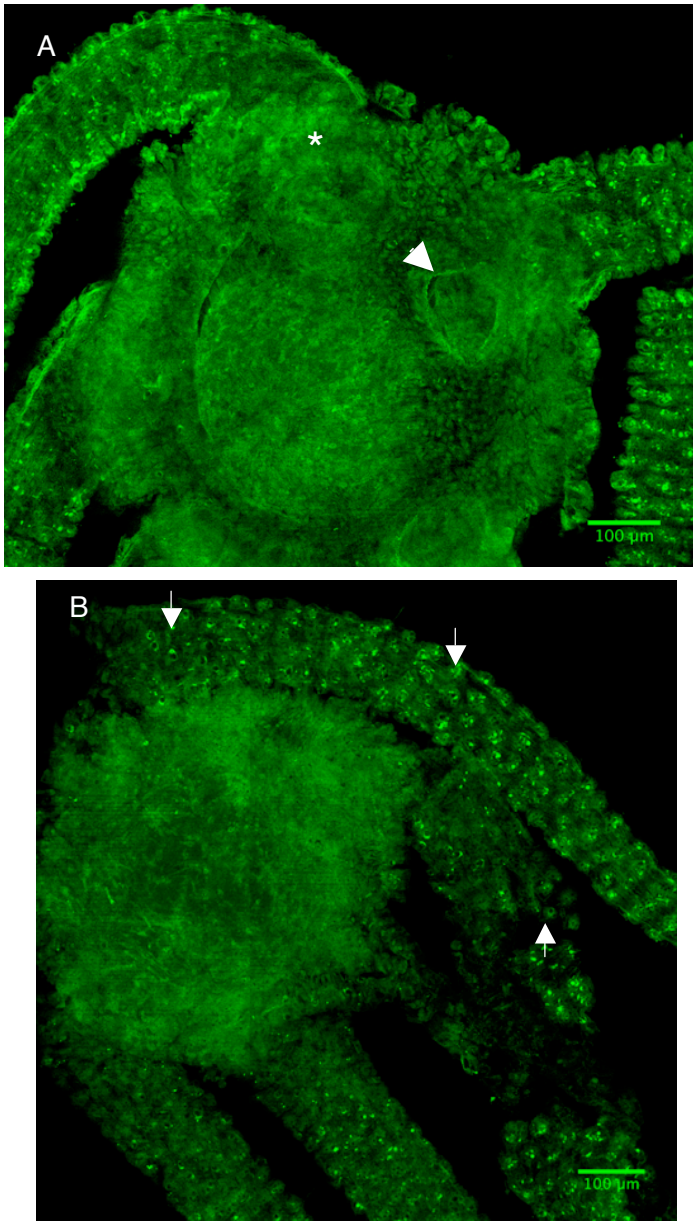

Figure 6. Anti-GABA$_B$ R1 rabbit polyclonal antibody.

A. Hypostome with attached tentacles (one tentacle (asterisk) is flipped over in hypostome). A circumtentacular nerve ring is visible (arrowhead). Size bar= 100 µm

B. Fluorescence is associated with nematocytes in battery cells in the tentacles (arrows). Size bar= 100 µm

Note: Preparations were double labeled with anti-GABA$_B$ R1 polyclonal antibody tagged with Alexa488 and anti-RFamide antibody tagged with TexasRed; due to technical difficulties we were unable to photograph anti-RFamide labeling.

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